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# Enhanced esterase gene expression and activity in a malathion-resistant strain of the tarnished plant bug, *Lygus lineolaris*

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**Abstract**

Extensive use of insecticides on cotton in the mid-South has prompted resistance development in the tarnished plant bug, *Lygus lineolaris* (Palisot de Beauvois). A field population of tarnished plant bugs in Mississippi with 11-fold higher resistance to malathion was used to examine how gene regulation conferred resistance to this organophosphate insecticide. In laboratory bioassays, synergism by the esterase inhibitors S,S,S,-tributylphosphorotrithioate (DEF) and triphenylphosphate (TPP) effectively abolished resistance and increased malathion toxicity by more than 80%. Esterase activities were compared in vitro between malathion susceptible and resistant (selected) strains. More than 6-, 3- and 10-fold higher activities were obtained with the resistant strain using $\alpha$-naphthyl acetate, $\beta$-naphthyl acetate, and *p*-nitrophenyl acetate, respectively. Up to 95% and 89% of the esterase activity in the susceptible and resistant strains, respectively, was inhibited by 1 mM DEF. Inhibition of esterase activity up to 75% and 85% in the susceptible and resistant strains, respectively, was obtained with 0.03 mM TPP. Esterase activities in field populations increased by up to 5.4-fold during the fall season. The increase was synchronized with movement of the insect into cotton where exposure to pesticides occurred. Esterase cDNA was cloned and sequenced from both malathion susceptible and resistant strains. The 1818-nucleotide cDNA contained a 1710-bp open reading frame coding a 570 amino acid protein which was similar to many insect esterases conferring organophosphate resistance. No amino acid substitution was observed between susceptible and resistant strains, indicating that esterase gene mutation was not involved in resistance development in the resistant strain in Mississippi. Further examination of esterase gene expression levels using quantitative RT-PCR revealed that the resistant strain had a 5.1-fold higher level of esterase mRNA than the susceptible strain. The results of this study indicated that up-regulation of the esterase gene appeared to be related to the development of resistance in the tarnished plant bug.
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*Keywords:* Malathion; Resistance; cDNA; mRNA; Expression; Esterase; Inhibitor; Tarnished plant bug; *Lygus lineolaris*

## 1. Introduction

The tarnished plant bug, *Lygus lineolaris* (Palisot de Beauvois), is an economically important pest of cotton in the mid-South. Currently, control of the tarnished plant bug relies exclusively on chemical insecticides. Resistance development in this insect is apparently associated with exposure to insecticides after they move from wild hosts into cotton fields.

Prior to their movement into cotton, tarnished plant bugs increase in number in the winter and spring on wild host plants found mainly in marginal areas near cultivated fields, ditches, and roads. Migration into cotton can occur from May to August, but the largest migration usually occurs during July and August as wild hosts mature and decline in number (Snodgrass et al., 1984). Plant bugs migrate from cotton back to wild hosts in the fall where they produce adults in diapause in September, October, and November which over-winter

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(Snodgrass, 2003). This pattern of movement into and out of cotton exposes plant bugs to insecticides and resistance development mainly during June, July, and August, but also allows for several generations to be produced outside of cotton on wild hosts with no selection pressure for insecticide resistance. However, during July and August a high percentage of the total population is in cotton where insecticide resistance can develop (Luttrell et al., 1998). A pattern for pyrethroid (Snodgrass, 1996a; Snodgrass and Scott, 2000) and malathion (Snodgrass and Scott, 2003) resistance development, where resistance levels were found in plant bugs collected from wild hosts in the spring, was usually significantly lower than resistance levels found in plant bugs collected from wild hosts at the same locations in the fall (after the cotton crop was mature).

Resistance to pyrethroid insecticides in tarnished plant bugs is currently widespread in the mid-South (Snodgrass and Scott, 2000). Insecticide resistance has reduced the effectiveness of chemical control and increased the cost and amount of insecticides needed to control this pest. Zhu and Snodgrass (2003) demonstrated different structures of cytochrome P450 cDNAs and corresponding gene expression differences between pyrethroid-susceptible and resistant strains. This P450 gene expression was inducible by pyrethroid treatment. Because of resistance development, pyrethroids are no longer recommended for plant bug control in cotton in Mississippi (Layton, 2003). There are currently 8 insecticides recommended for control of plant bugs in cotton in Mississippi, and five of these insecticides (acephate, dicrotophos, malathion, methamidophos, and profenophos) are organophosphates. Acephate, dicrotophos, and malathion are the most widely used organophosphates for plant bug control. Multiple applications of malathion to cotton, done for boll weevil eradication in Mississippi during 1999–2001, were found to cause increases in malathion resistance as high as 30.5-fold in plant bug populations collected from wild hosts in the fall after the malathion treatments to cotton ended (Snodgrass and Scott, 2003).

Increased economic importance and development of resistance to both pyrethroids and organophosphates has prompted research to understand the mechanisms of the resistance in this insect. Malathion resistance is usually associated with increased carboxylesterase activity (Liu and Han, 2003; Wool and Front, 2003; Perez-Mendoza et al., 2000; Smyth et al., 2000; Raghavendra et al., 1998). Single-gene point mutation was also observed in many resistant insects (Campbell et al., 1998; Zhu et al., 1999; Smyth et al., 2000). Resistant insects were able to survive malathion treatment by sequestering (Karunaratne and Hemingway, 2001) or metabolizing malathion to non-toxic malathion acids and malaoxon acids (Campbell et al., 1998). However, very little research has been done to characterize molecular and genetic basis of the malathion resistance in the tarnished plant bug. In this study, we used a malathion-resistant population to begin investigations into mechanisms leading to the resistance, since knowledge of biochemical characteristics and gene regulation are very useful in the monitoring and management of insecticide resistance. Here, we report the first malathion-resistance-related esterase cDNA from both susceptible and resistant strains of the tarnished plant bug. Esterase enzyme activities, inhibition, and gene transcript expression levels were quantitatively analyzed and compared between the 2 strains.

## 2. Materials and methods

### 2.1. Insect

The malathion-susceptible strain was collected from weeds near Crossett, AR (Ashley County). Cotton is not grown near Crossett and plant bugs from this location are very susceptible to most insecticides used on cotton (Snodgrass, 1996b). The resistant strain was collected near Mound Bayou (Bolivar County), MS. Field collected insects were used for bioassay, and malathion-selected insects were used for enzyme activity assay and molecular analyses.

### 2.2. Bioassay and resistance levels

A glass-vial bioassay (Snodgrass, 1996b) was used to determine resistance levels in the two insect populations. Adult plant bugs were placed into 20-ml glass scintillation vials (two insects per vial) that had been treated with the insecticide being studied. Each insecticide was applied by pipetting 0.5 ml of the insecticide diluted in acetone into each vial. Each vial was rolled on its side until an even layer of insecticide dried on its inner surface. Vials in control treatments received only 0.5 ml of acetone, and in all tests insecticides were applied to the vials on the same day that the test was performed. A small piece of green bean pod, *Phaseolus vulgaris* L., (cut transversely) about 3 mm thick was added to each vial as food for the adults, and a cotton ball was used to seal each vial. Technical-grade insecticides, acephate, dicrotophos, malathion, and permethrin, used in the tests were purchased from Chem Service (West Chester, PA), and were all $\geqslant 98\%$ pure.

Vials were held during a test in an upright position at laboratory conditions of 24–26 °C, and humidity was not controlled. Mortality was determined after 24 h, and adults were considered dead if they were unable to right themselves or walk, or there was no movement when they were prodded. All bioassays had 3 replications of 6–9 different insecticide concentrations, and in each replication of each concentration included 5 vials with 2

adults. Data were corrected for control mortality using Abbott's (1925) formula before analysis. Data were analyzed with the PROC Probit option of SAS (SAS Institute, 1997). Differences in $LC_{50}$ values were considered significant if the 95% CL of the resistance ratio at the $LC_{50}$ level did not include 1.0 (Robertson and Priesler, 1992).

The field-collected adults from Mound Bayou were selected with malathion to produce a more homozygous malathion-resistant test population for enzyme activity assays and molecular analyses. Adults were placed into 20-ml glass vials (2 adults per vial) that had been treated with 200 µg of malathion and exposed for a 3-h period. Survivors were removed from the vials and used in the enzyme assays and molecular analyses. Synergists were tested in the glass vials by exposing adults (2 per vial) to 50 µg/vial TPP or 100 µg/vial DEF for 1 h. Adults were then transferred to vials (2 per vial) treated with 200 µg/vial malathion. Mortality was recorded after 3 h.

### 2.3. Enzyme preparation

Individual tarnished plant bugs were homogenized in 340 µl 0.1 M sodium phosphate buffer, pH 8.0. Homogenates were then centrifuged at 10,000*g* for 5 min at 4 °C, and the supernatant was used for enzyme analysis. Protein concentrations were determined using the Pierce (Rockford, IL) protein assay kit. Bovine serum albumin was used to titrate the standard curve.

### 2.4. Esterase assays

To determine esterase activity, microtiter plate assays were conducted using the following substrates: PNPA (*p*-nitrophenyl acetate), α- and β-naphthyl acetate. PNPA was added to 200 µl diluted enzyme homogenate (20 ×) to obtain a final concentration of 0.5 mM. A Bio-Tek $EL_x808_{iu}$ was used to monitor the assay at 405 nm for 10 min with readings taken every 15 s (Rose et al., 1995). The following substrate solution was made fresh before each assay with α- and β-naphthyl acetate: 9 mg Fast Blue Salt, 300 µl of 0.113 M α- or β-naphthyl acetate, and 15 ml of 0.1 M phosphate buffer, pH 7.0. The substrate solution was vacuum filtered once through Whatman 52 filter paper, and 240 µl of substrate solution was added to 10 µl of diluted enzyme homogenate to start the reaction. The assay was monitored at 450 nm for 10 min with measurements every 15 s (Ottea et al., 2000). Inhibitors for esterase activity were DEF with final concentration of 1 mM (Park, 1999) and TPP with final concentration of 0.03 mM (Smyth et al., 1996). Inhibitors were added to enzyme homogenate and allowed to incubate at 37 °C for 10 min before the addition of substrate (Park, 1999). The enzyme activity assays were conducted 10 times with different insect samples from the susceptible and the malathion-selected strains. Average of mean velocity and standard error were calculated using all 10 values determined for each substrate tested.

### 2.5. Seasonal survey of esterase activities

Through the months of May–October of 2003, tarnished plant bugs were collected from weeds in Sunflower County, Mississippi. Ten individual adults for each month were tested in duplicate for levels of esterase activity as previously described.

### 2.6. RNA extraction and cDNA synthesis

Five tarnished plant bug adults (approximately 30 mg) were homogenized in 500 µl TRIZol solution (Invitrogen, Carlsbad, CA). Total RNA was precipitated with isopropanol and resuspended in double-distilled $H_2O$. Five microgram of RNA was used as a template for cDNA synthesis using an oligo-dT primer in the reverse transcription (RT) reaction (SuperScript First Strand cDNA Synthesis System, Invitrogen, Carlsbad, CA). The procedures were performed using insects from the susceptible strain and repeated using insects from the malathion-selected strain.

### 2.7. Cloning esterase cDNA

To clone esterase cDNA fragments, two degenerate forward primers and one degenerate reverse primer were designed from three conserved regions (amino acid sequences: DDCLY(H)LNV, GNAGLKDQ, and FGESAGG) of carboxylesterases from insects and rats (Field et al., 1993; Tittiger and Walker, 1997; Vaughan et al., 1997; Yan et al., 1994; Zhu et al., 1999). PCR amplification of genomic DNA resulted in a 462-bp fragment, part of which matched the esterase coding region. Two specific primers, LLEstF1 and LLEstF2 (Fig. 4), were designed from cloned DNA sequence, and were used with oligo-dT primer to amplify 3′-end cDNA. To obtain the 5′-end of the esterase cDNA, three reverse primers, LLEstR1, LLEstR2, and LLEstR3 (Fig. 4), were synthesized. Reverse transcription was performed using the reverse primer LLEstR1, and the 5′-end of esterase cDNA was isolated and C-tailed using the 5′-RACE (rapid amplification of cDNA end) system (Invitrogen). Subsequent semi-nested amplifications were performed using a forward abridged anchor primer (5′-RACE kit) and the specific reverse primers LLEstR2 and LLEstR3. The DNA fragment resolved from the 5′-RACE amplification was cloned into a pGEM-T vector and sequenced from both directions. Esterase cDNA was assembled, and it was confirmed that it contained the full coding sequence for the esterase-like protein.

To obtain near full-esterase-like cDNA from the susceptible and malathion-selected strains of the tarnished plant bug, total RNA was extracted from these strains and used for RT-cDNA synthesis. A forward primer LLEstF0 and a reverse primer LLEstR0 were used in PCR reactions to flank the 5′ and 3′ non-coding regions, respectively (Fig. 4). To eliminate potential error created by Taq DNA polymerase, a thermal-stable proof-reading Pfu DNA polymerase (Promega, Madison, WI) was used to reamplify a near full-length esterase cDNA from the RT-cDNAs of the susceptible and resistant strains. The resolved near full-length cDNA fragments (~1.8 kb) were purified from low melting point agarose gel, and then A-tailed for 30 min at 72 °C using the nucleotide adenine and Taq DNA polymerase. They were then cloned into a pGEM-T vector (Promega), and the resolved clones were sequenced from both directions on an ABI 3700 sequencer.

The BLASTX program was used to search the sequence database of the National Center for Biotechnology Information Internet server for proteins with amino acid sequence similarity to the esterases (Altschul et al., 1997). The proteomics tools of the ExPASy Molecular Biology Server of Swiss Institute of Bioinformatics (http://www.expasy.ch/tools/) were used to process data of the deduced protein sequences. The ClustalW method (Thompson et al., 1994) on an Internet Server (http://pbil.ibcp.fr) was used to conduct multiple sequence alignment.

### 2.8. Quantitative reverse transcription (QRT) PCR analysis of esterase mRNA expression

The expression levels of esterase mRNAs were determined quantitatively in the susceptible and resistant strains using procedures modified from those used by Alexandre et al. (1998), Guenthner and Hart (1998), Igaz et al. (1998), Freeman et al. (1999), and Zhu et al. (2000). To develop a homogeneous internal standard for QRT-PCR analysis of esterase mRNA expression, the 5′-end cDNA fragment was amplified from plasmid DNA using the forward primer LLEstF0 and a reverse primer LLEstR3 (*Hind III* sequence was attached). The 3′-end cDNA fragment was amplified using a forward primer LLEstF4 (*Hind III* sequence was attached) and a reverse primer LLEstR0. Two cDNA fragments were digested separately with restriction enzyme *Hind III*, gel-purified, and ligated using T4 DNA ligase (Promega). The ligated cDNA fragment was identified and purified from low melting agarose gel, and ligated into a p-GEM-T vector. The resolved clone was verified for the presence and correct orientation of both 5′ and 3′-ends of the esterase-like cDNA with 259-bp deletion by PCR and agarose gel electrophoresis. It was subsequently used as the internal standard for QRT-PCR analysis of esterase-like mRNA expression as described below.

RNA was prepared from malathion-selected and susceptible plant bugs. The concentration of the RNA was quantified using a spectrophotometer. Reverse transcription was conducted with an oligo-dT primer and SuperScript II reverse transcriptase (Invitrogen). Five microgram of total RNA from each sample was used to generate first strand cDNA. QRT-PCR was conducted six times with different RNA preparations for each strain. Specific primers LLEstF1 and LLEstR4 (Fig. 4) were used to simultaneously amplify a 684-bp esterase cDNA fragment from the target cDNA template and a 425-bp cDNA fragment from the internal standard. The concentration range of the internal standard was determined by preliminary experiments. The internal standard was diluted in distilled water as follows: 0.2, 0.5, 1, 2, 5, 10, 20, and 50 pg/μl to match target mRNA levels, and these were used in the QRT-PCR reactions. One microliter of standard and 0.25 μL of target cDNA (equivalent to 0.0625 μg of total RNA) were added to the PCR reaction mixture. PCR products were separated on 1% agarose gels, stained with 0.5 μg/mL ethidium bromide, and photographed under UV light using a Kodak 290 digital camera equipped with 1D software. Band intensity was determined by using Kodak 1D image analysis software (Version 3.54). The relationship between esterase mRNA concentrations and PCR band intensity was determined by plotting the log values of band intensity against the log values of the standard concentrations using Sigma Plot.

## 3. Results

### 3.1. Resistance levels to insecticides

Tarnished plant bugs collected near Mound Bayou, MS had significantly higher levels of resistance to all insecticides tested, as compared to plant bugs collected from Crossett AR (Table 1). The highest resistance ratio between the two populations was obtained with malathion. The lethal concentration to achieve 50% mortality was 4.13 μg/vial for the susceptible population from Crossett. It required an 11-fold higher concentration of malathion to achieve the same mortality rate in the resistant population from Mound Bayou. The malathion-resistant population from Mound Bayou also had multiple resistance to a pyrethroid insecticide, Permethrin (Table 1).

### 3.2. Synergists and malathion toxicity

TPP and DEF had very low toxicity to the malathion-resistant plant bugs. Approximately 1.7% and 5% of

Table 1
Morality of malathion-resistant adult tarnished plant bugs from Mound Bayou, MS and susceptible tarnished plant bugs from Crossett, AR exposed to four insecticides in a glass-vial bioassay

| Insecticides | Mound Bayou, MS | | Crosset, AR | | $RR_{50}$[b] |
|---|---|---|---|---|---|
| | Slope±SE | $LC_{50}$[a] | Slope±SE | $LC_{50}$ | |
| Permethrin | 1.25±0.21 | 15.75 | 1.01±0.08 | 3.39 | 4.5 |
| Dicrotophos | 1.05±0.15 | 6.98 | 1.44±0.18 | 1.62 | 4.1 |
| Acephate | 1.98±0.17 | 10.24 | 1.76±0.20 | 3.10 | 3.3 |
| Malathion | 1.51±0.08 | 45.88 | 1.29±0.17 | 4.13 | 11.1 |

[a]Lethal concentration ($LC_{50}$: μg/vial) for achieving 50% mortality at 24 h.
[b]Resistance ratio (RR): ratio of $LC_{50}$ for Mound Bayou to $LC_{50}$ for Crossett.

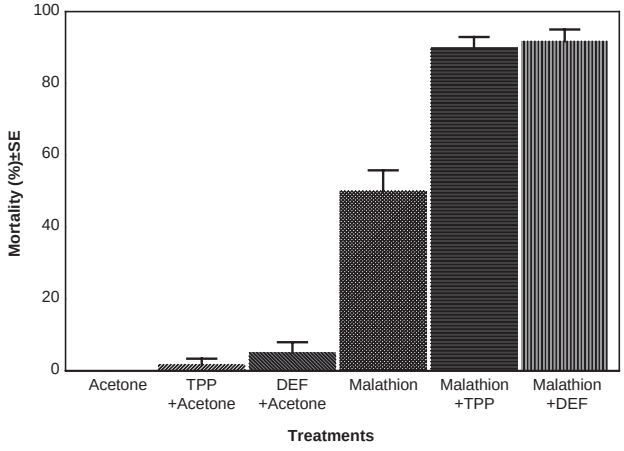


Fig. 1. Mortality of malathion-resistant tarnished plant bug adults treated with malathion only or combination of malathion and esterase inhibitors (DEF or TPP).

adults were killed by TPP and DEF, respectively (Fig. 1). Malathion at a rate of 200 μg/vial killed 50% of the tarnished plant bug adults. The mortality rates for the same concentration of malathion were increased to 90% and 92% after the insects were pre-treated with TPP and DEF (Fig. 1).

### 3.3. Esterase activity and inhibition

Three esterase substrates and two inhibitors were used to compare esterase activity and inhibition between malathion-susceptible and resistant (selected) strains of the tarnished plant bug. The resistant strain had 6.2-fold higher α-naphthyl acetate activity than the susceptible strain. Up to 95% and 75% of the esterase activities in the susceptible strain were inhibited by 1 mM DEF or 0.03 mM TPP. More than 71% and 85% of the esterase activities in the resistant strain were suppressed by DEF and TPP (Fig. 2A). The resistant strain had 3-fold higher β-naphthyl acetate activity than the susceptible

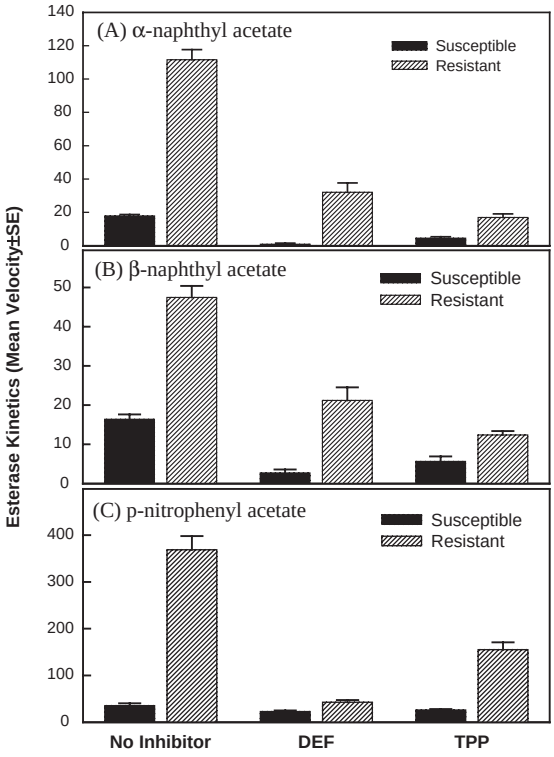


Fig. 2. Comparison of esterase activities between malathion-susceptible and resistant strains of the tarnished plant bug, and suppression of activities by two esterase inhibitors (DEF and TPP) on three substrates. (A) α-naphthyl acetate; (B) β-naphthyl acetate; (C) *p*-nitrohenyl acetate.

strain. Up to 84% and 66% of the esterase activities in the susceptible strain were inhibited by DEF and TPP, respectively. Approximately 56% and 74% of the esterase activities in the resistant strain were suppressed by DEF and TPP, respectively (Fig. 2B). The resistant strain had 10.4-fold higher *p*-nitrophenyl acetate activity than the susceptible strain. Only 36% and 26% of the esterase activities in the susceptible strain were inhibited by DEF and TPP. More than 89% and 59% of the esterase activities in the resistant strain were suppressed by DEF and TPP (Fig. 2C).

### 3.4. Seasonal variation in esterase activity

Results from measuring esterase activity in adult plant bugs collected each month in Sunflower County demonstrated that the esterase activity increased as the season progressed (Fig. 3). The α-naphthyl acetate activity was low from May to July, and then increased to a 4.8-fold higher level in October. The β-naphthyl acetate activity showed a similar trend of increase, with more than 3.4-fold higher activity in October as compared to July. The *p*-nitrophenyl acetate activity remained relatively low before August. A sharp increase in activity was observed during the period from August to October. In October, the activity reached a 5.4-fold

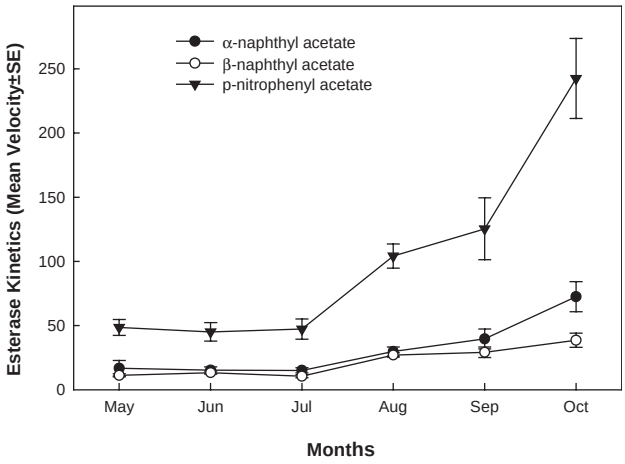


Fig. 3. Seasonal variation of esterase activities in the tarnished plant bugs collected in Sunflower County, Mississippi. Enzyme activities were determined on three substrates: α-naphthyl acetate, β-naphthyl acetate and *p*-nitrohenyl acetate.

higher level than that observed in the early seasons (Fig. 3).

### 3.5. Esterase cDNA sequence

Both 5′ and 3′-ends of esterase-like cDNA were successfully cloned. Forward primer LLEstF0 and reverse primer LLEstR0 were designed (Fig. 4), and were used to amplify near full-length esterase cDNA with pfu DNA polymerase. Three esterase cDNA clones were sequenced from the susceptible and malathion resistant (selected) strains. Sequence alignment indicated that the esterase cDNAs from both strains had identical sequences.

Esterase cDNA cloned from the tarnished plant bug contained 1918 nucleotides. A start codon, ATG, was located at positions 54–56, and a termination codon, TGA, was located at positions 1764–1766 (Fig. 4). The open reading frame covered 1710 nucleotides which translated to 570 amino acid residues (Fig. 4).

### 3.6. Deduced esterase

The predicted esterase-like protein consisted of 48 positively charged residues (Arg + Lys), 59 negatively charged residues (Asp + Glu) with a molecular mass of 63.39 kDa and a theoretical pI of 5.78. The SignalP program (Nielsen et al., 1997) predicted a 17-amino acid residue signal peptide with a likely cleavage site between residue 17 (alanine) and residue 18 (glutamic acid).

The homology search of GenBank using BlastP revealed that the predicted 570-residue protein was similar to several insect esterases which are responsible for organophosphate resistance development. Six esterase sequences were downloaded from the GenBank. These sequences included an Esterase FE4 precursor from the green peach aphid *Myzus persicae* (P35502, Field et al., 1993), an esterase from the honey bee *Apis mellifera* (BAC54130, submitted by Kamikouchi et al.), an esterase-like protein from *Anopheles gambiae* (XP_312564, Celera Genomics, Rockville, MD), an esterase-like protein from the fruit fly *Drosophila melanogaster* (AAM29421, submitted by Lawrence Berkeley National Laboratory, Berkeley, CA), a carboxylesterase precursor from the brown planthopper *Nilaparvata lugens* (AAG40239, Small and Hemingway, 2000), and a carboxylesterase from *Anisopteromalus calandrae* (AAC36245, Zhu et al., 1999).

The six esterase sequences downloaded from GenBank were aligned with the sequence obtained from the tarnished plant bug (Fig. 5) using the ClustalW method (GapOpen = 5, GapExt = 0.5). Overall sequence identity among these 7 esterases was only 10%. The esterase sequence from the tarnished plant bug was subjected to pairwise sequence comparison to the other 6 insect esterases. Results showed that the sequence identities ranged from 29.41% (AAC36245) to 33.85% (BAC54130). Three residues, serine, glutamic acid, and histidine (which form a catalytic center), were conserved among the 7 insect esterases (Fig. 5).

### 3.7. Esterase mRNA expression levels

By using the specific primers LLEstF1 and LLEstR4, the RT-PCR was successful in simultaneously amplifying a 684-bp esterase cDNA fragment from target cDNA template and a 425-bp cDNA fragment from the internal standard. Both cDNA fragments were clearly separated on agarose gel (Fig. 6A). Band intensity of the 425-bp internal standard decreased as its concentration decreased, while the band intensity of the 684-bp target cDNA fragment increased as the internal standard concentration decreased. Fig. 6B was a representative of the relationship between esterase mRNA concentrations and PCR band intensity. The crossing point of the two lines was the point at which the target mRNA and the internal standard were equal. Means of the esterase mRNA concentrations were calculated from 6 QRT-PCR measurements for each strain. Results (Fig. 6C) showed that the malathion-susceptible strain had $32.8 \pm 10.3$, and the resistant strain had $165.9 \pm 93.3$ pg of esterase mRNA per microgram of total RNA. The resistant strain had significantly higher (5.1-fold, $P < 0.05$) esterase mRNA levels than the susceptible stain.

## 4. Discussion

Organophosphate insecticides have been used for more than 25 years in the mid-South to control tarnished plant bugs in cotton. The overall trend in

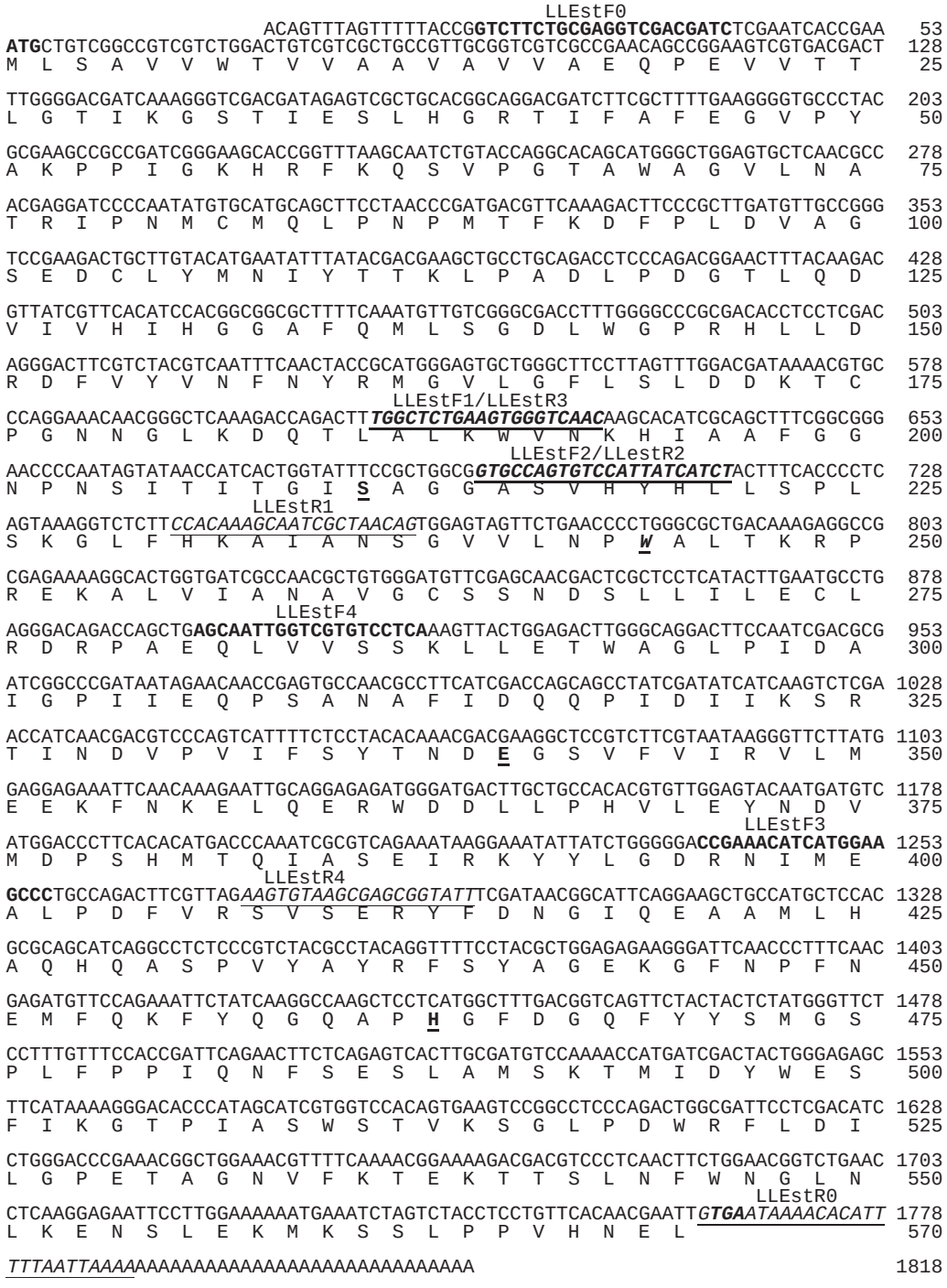


Fig. 4. Nucleotide and predicted amino acid sequences of esterase isolated from *L. lineolaris*: **ATG** = start codon, **TGA** = termination codon. Forward primer sequences used for cloning and sequencing cDNA are showed as bold letters and labeled on the top. Complementary sequences of the reverse primers are indicated by italic and underlined letters. The bold and underlined letters **S**, **E** and **H** are three catalytic center residues. The sequences have been deposited in GenBank with accession number AY538666.

control with organophosphate insecticides is for reduced efficacy (Howell et al., 1998). Part of the reduced efficacy is due to widespread pyrethroid-resistance in the mid-South (Snodgrass and Scott, 2000), since pyrethroid-resistant plant bugs also have increased tolerance to several organophosphate insecticides (Snodgrass, 1996a). This cross-resistance may be similar to the pyrethroid-resistance in *Blatella germanica*, which was significantly correlated with the level of general esterase activity (Valles, 1998). Pyrethroid insecticides are still recommended and used in cotton, in the mid-South, to control various lepidopteran pests. The continued use in

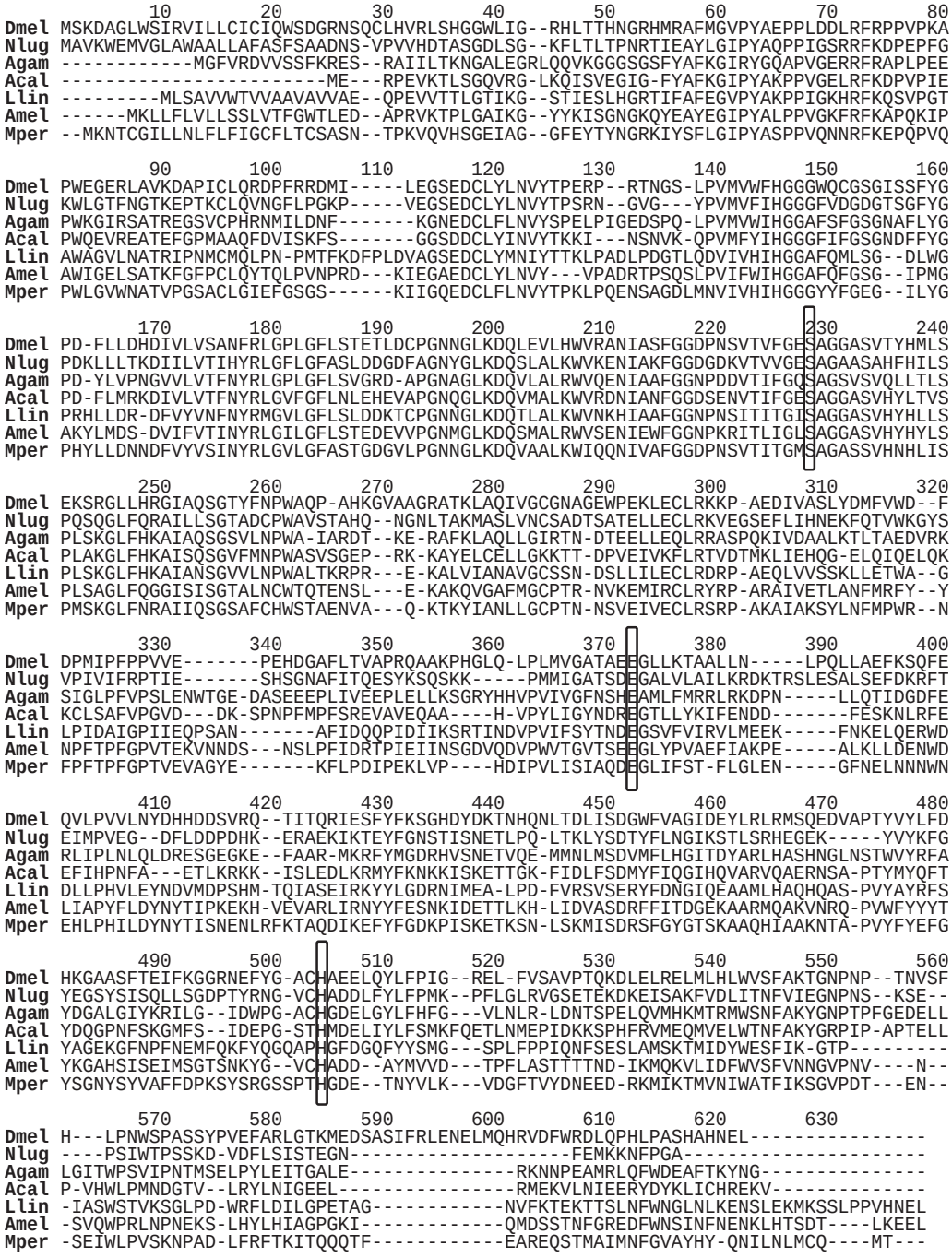


Fig. 5. Predicted amino acid sequence **Llin** (AY538666) of esterase from *L. lineolaris* and alignment with 6 other insect esterase sequences. **Dmel**: an esterase-like protein from *D. melanogaster* (AAM29421), **Nlug**: a carboxylesterase precursor from *N. lugens* (AAG40239), **Agam**: an esterase-like protein from *A. gambiae* (XP_312564), **Acal**: a carboxylesterase from *A. calandrae* (AAC36245), **Amel**: an esterase from *A. mellifera* (BAC54130), **Mper**: an Esterase FE4 precursor from *M. persicae* (P35502). Three catalytic center residues were boxed. Hyphens represent sequence alignment gaps.

cotton exposes plant bugs to pyrethroids and helps to maintain their pyrethroid-resistance. Some populations of a closely related species, *L. hesperus* Knight found in the Western United States, have developed high levels of resistance to the organophosphate trichlorfon. This resistance is caused by enhanced esterase detoxification (Zhu and Brindley, 1990a) and reduced acetylcholinesterase sensitivity (Zhu and Brindley, 1990b). The tarnished plant bug population we studied from Mound Bayou, MS, was collected from weeds in May of 2003 and had significantly higher resistance (as compared to susceptible bugs from Crossett, AR) to a pyrethroid and three organophosphate insecticides. The highest resistance was to malathion (11-fold higher in resistant than

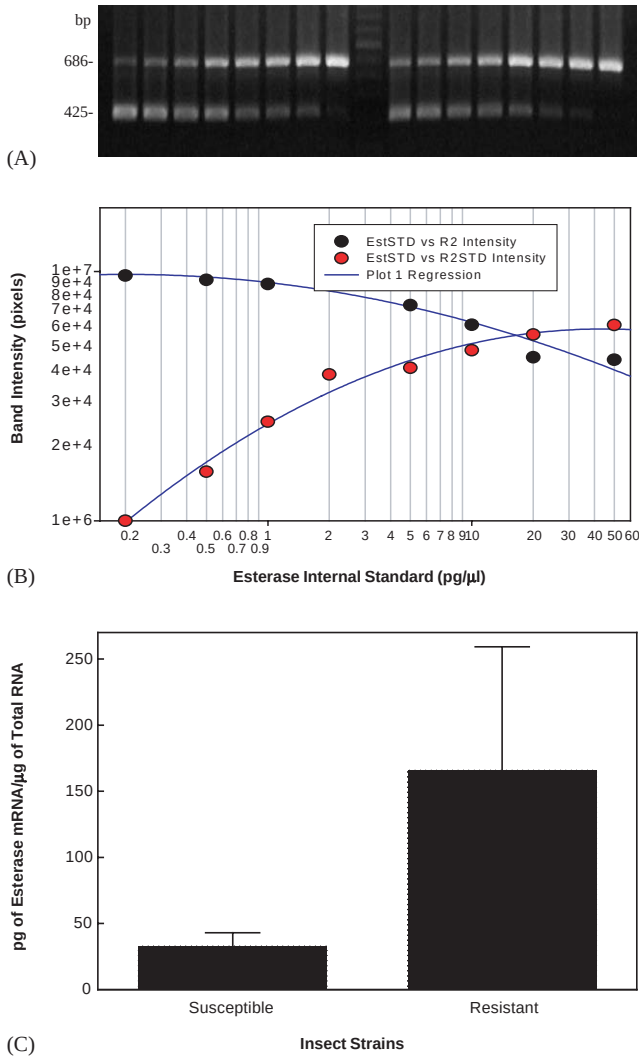


Fig. 6. (A) Representative electrophoretic graph of QRT-PCR conducted in 8 tubes incorporated with internal standard from high to low concentration (lower band). The target cDNA band intensity (upper band) increased as the internal standard concentration decreased. (B) Representative relationship between band intensity and concentration of internal standard. Intensity of each band was determined by using Kodak 1D software. (C) Quantitative RT-PCR analyses of esterase mRNA (pg/µg of total RNA) in malathion-susceptible and resistant strains of *L. lineolaris*.

in susceptible population) which suggested that these plant bug populations had been exposed to malathion in cotton during the growing season in 2002. It is likely that the level of resistance to malathion was higher in these plant bugs during the fall of 2002 when the plant bugs migrated from cotton to wild hosts. Snodgrass and Scott (2003) tested plant bug populations in Mississippi for resistance to malathion and found resistance as high as 30-fold in the fall during boll weevil eradication in 1999–2001. At nearly every location in which plant bugs were found with elevated malathion-resistance in the fall, the resistance had declined significantly when plant bugs from the same location were tested in the spring of the following year. The reasons for the decline were not determined.

In our study, we found that resistance development to malathion was highly associated with increased esterase activity which indicated that metabolic detoxification was very likely the major resistance mechanism. Two esterase inhibitors, DEF and TPP, significantly suppressed esterase activity (Fig. 2), which has been shown to play a major role in malathion detoxification in insects (Campbell et al., 1998; Field et al., 1993; Newcomb et al., 1997: Wool and Front, 2003; Zhu et al., 1999). In addition, both esterase inhibitors greatly increased the toxicity of malathion for the resistant plant bugs (Fig. 1). Increased toxicity to the tarnished plant bug might be attributed to the inhibition of the detoxifying enzymes by DEF and TPP, a theory supported in this study in which up to 95% and 85% of the esterase activity was suppressed by DEF and TPP, respectively. We also found that esterase activity increased during the growing season (Fig. 3), and was synchronized with the exposure of plant bugs in cotton to insecticides. This finding is similar to several cases of seasonal variation in insecticide resistance in other insects (Daly and Fisk, 1993; Mangel and Plant, 1983; Lenormand et al., 1999). Exposure of a population to insecticides kills the susceptible members of the population, and resistance levels can increase as the number of resistant individuals increases (Mallet, 1989; Mani and Wood, 1984).

Because metabolic detoxification was very likely the major resistance mechanism in the tarnished plant bug, we further examined esterase cDNA structures and gene expression levels between the malathion susceptible and resistant strains. The open reading frame of the esterase-like cDNA from the tarnished plant bug encoded a protein of 570 amino acid residues. Topology prediction indicated that no transmembrane helix was found for the putative esterase-like protein. The predicted protein sequence included 3 potential *N*-glycosylation sites at positions 74–77, 266–269, and 483–486, and a carboxylesterase type-B serine active site FGGNPNSITITGISAG at positions 198–213 (Fig. 4).

In many resistance cases, gene mutation is involved in resistance development. A relatively large amino acid, tryptophan, is located near a catalytic center. Mutation to a smaller residue (Campbell et al., 1998; Zhu et al., 1999) might create more space around the catalytic serine which would reduce steric hindrance of the inversion about the phosphorus atom that must occur when the serine–phosphorus bond is hydrolyzed. (Campbell et al., 1998). In the tarnished plant bug, gene mutation is not involved in resistance development because we cloned esterase cDNAs with identical sequence from both susceptible and resistant strains. The tryptophan at position 244 (Fig. 4) was retained in

both strains, and this residue was conserved for all 7 selected esterases (Fig. 5).

We examined esterase gene expression levels between the susceptible and resistant strains. Transcriptional overproduction by an esterase gene has been observed in many insects which are resistant to organophosphates (Berrada and Fournier, 1997; Field et al., 1999; Zhu et al., 1999; Harold and Ottea, 2000). In this study, we found that the resistant strain of the tarnished plant bug had a 5.1-fold higher level of esterase gene transcript than the susceptible strain. Overproduction of gene transcripts can result from up-regulation of the transcription of a single copy of the esterase gene (Berrada and Fournier, 1997) or accumulation of multiple copies of the esterase genes (Hawkes and Hemingway, 2002; Siegfried et al., 1997). Elevated levels of esterase gene expression may reach up to 30-fold (Zhu et al., 1999) or range from 4 to 80-fold higher (Field et al., 1999) in resistant strains. Esterases are often found in multigene families (Oakeshott et al., 1993; Robin et al., 1996) and it is very likely that a complex of esterases is present in the tarnished plant bug. Although we do not know whether the tarnished plant bug has multiple esterase genes, the esterase cDNA reported here should exactly represent the esterase gene that is overexpressed in the resistant strain. Unlike Northern blotting, cross reaction is greatly reduced in our quantitative RT-PCR system. The specificity is achieved through specific binding regions on the esterase cDNA for specific primers used in the quantitative RT-PCR. Using this technique, we were able to directly link the overexpressed gene to resistance development in the tarnished plant bug. Increased transcription in the resistant strain may be the underlying cause of esterase-mediated insecticide resistance in the tarnished plant bug. However, other metabolic mechanisms, such as oxidases and glutathione *S*-transferase, have not been excluded.

In summary, this study provides information on resistance gene regulation in malathion-resistant strains of the tarnished plant bug. Esterase-mediated metabolic resistance resulted in more than 10-fold increase in esterase activity in a resistant strain conferring more than an 11-fold increase in resistance to malathion in a natural population. Increased esterase gene expression levels may be responsible for the resistance development. Two esterase inhibitors exhibited significant suppression to esterase activities, and synergism for malathion. Future studies will include molecular cloning of other members of the esterase gene family and investigation of regulatory factors for gene overexpression. Other resistant populations will be examined, and genetic linkage between resistant phenotypes and related genes, including esterase genes, will be established. Molecular or biochemical markers will be developed to monitor resistance in field populations and to provide information for developing pest management strategies.

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